Cirrhosis is associated with lower serological responses to COVID-19 vaccines in patients with chronic liver disease

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Supplementary patients and methods

Study population

Recruitment centers included the C.U.B. Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium; La Fe University Hospital, University of Valencia, Valencia, Spain; Medical University of Graz, Graz, Austria; Azienda Ospedale-Università Padova, Padova, Italy; "Grigore T. Popa" University of Medicine and Pharmacy, "St. Spiridon" Emergency Hospital, Iasi, Romania; Centro Hospitalar Universitário Lisboa Norte, Lisbon, Portugal; Virgen del Rocío University Hospital, Institute of Biomedicine of Seville, University of Seville, Spain.

Patients were grouped according to disease etiology into viral hepatitis (HBV, HCV, HDV and/or HEV), autoimmune and/or cholestatic liver disease (primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC) and/or autoimmune hepatitis (AIH)), metabolic related fatty liver disease (MRFLD; including patients with non-alcoholic fatty liver disease (NAFLD) and/or heavy alcohol consumption), hereditary liver disease (Wilson disease, hemochromatosis and polycystic liver disease), and cryptogenic liver disease. Pharmacology was grouped into immunosuppressive treatment (Prednisone, Tacrolimus, Mycophenolate mofetil, Azathioprine, Budesonide and/or other), antiviral therapy (Tenofovir, Entecavir, Interferon, and/or other), and metabolic therapy (ursodeoxycholic acid, obeticholic acid, fibrates, metformin, GLP-1 agonists, insulin, and/or other). Liver disease stage was categorized according to the Child-Pugh class into CLD without cirrhosis, cirrhosis Child-

Pugh class A, cirrhosis Child-Pugh class B, and cirrhosis Child-Pugh class C. Additionally, comorbidities (type II diabetes, obesity, others) were also recorded.

Measurement of antibodies

SARS-CoV-2 spike S1 and RBD proteins were used immobilized onto high binding polystyrene 384 well microplates (Corning), overnight at 4ºC, diluted at 1.00 µg/mL in phosphate buffer saline (PBS) at pH 7.4. On the following day, plates were washed once with 100 µL/well of Wash Buffer (PBS pH 7.2, containing 0.05% Tween- 20), to remove unbound proteins. Plates were then blocked, for 2 h at 37ºC, with 3% BSA dissolved in PBS containing 0.1% Tween-20, to prevent unspecific binding of proteins to the plate. After washing, diluted serum samples were added in duplicate to plates and incubated under gentle agitation for 1 h at 24ºC. Following a washing cycle, goat anti-human IgG Fc horseradish peroxidase (HRP) conjugated (Invitrogen) and/or goat anti-human IgM µ chain HRP conjugated (Abcam) were added at a 1:20 000 dilution and 1:250 000, respectively, and incubated for 1 h at 24ºC. After washing, the signal was developed using an ultrasensitive TMB substrate. The reaction was stopped after 10 minutes with 0.5 M sulfuric acid and optical density (OD) values were read at 450 nm using a plate reader (VarioSkan Lux, ThermoFisher Scientific). Antibody levels were quantified through a calibration curve using a serum sample with previously determined antibody count using Fluidity One-W Serum system from Fluidic Analytics. The cut-off point - to establish positivity - was calculated using a mean value of the concentration of a cohort of 45 pre-pandemic negative

controls, plus three times their standard deviation. The threshold was defined as 7 nM and 1.22 nM for the IgG and IgM assays, respectively.

Surrogate neutralization assay

Biotinylated human ACE2, His, avitag™ (AC2-H82E6) and SARS-CoV-2 Spike (S) protein RBD, mouse IgG2a Fc (SPD-C5259, AcroBiosystems) were reconstituted according to the manufacturer´s instructions. All reagents were diluted in AlphaLISA Hiblock buffer 10-fold diluted (1x) (AL004C, PerkinElmer) and the assay was performed according to non-wash two step procedure. First, AlphaLISA® anti-mouse IgG coupled to acceptor beads (AL105C, Perkin Elmer) was diluted at 50 µg/mL (5x final concentration) and mixed along with the reagents diluted at 25 nM and 50 nM respectively (20x final concentration) in 30% of the final reaction volume (v/v). Next, we distributed on each well, a volume of 7.5 µL of this solution into a grey light AlphaPlate™-384 (6005350, Perkin Elmer). Plasma samples previously diluted in 1% BSA in [PBS-t] (1:10 final concentration) were added to well plates in duplicated. Assay plates were sealed and incubated for 1 h at 24ºC with agitation at 600 rpm. Second, AlphaScreen® Streptavidin Donor Beads (6760002, Perkin Elmer), was previously diluted at 40 µg/mL (2x final concentration) in 50% of the final reaction volume (v/v) and a volume of 12.5 µLof this solution was distributed on each well. Plates were sealed again and incubated 1 h plus at 24ºC with agitation at 600 rpm and protected from light. Finally, plates were read on a Varioskan™ LUX multimode microplate reader (ThermoFisher Scientific) with filter emission settings for 615/620 ηm. Percentage of neutralization was calculated as follows: [1-(mean Alpha signal from unknown

plasma samples/mean Alpha signal from negative plasma samples)*100] (Cao Y et al., Cell 2020).

For determining the optimal cut-off (Unal I, Comput Math Methods Med 2017), sera samples from healthy volunteers, screened for SARS-CoV-2 Nucleocapsid and Spike proteins, were used (n=78). The test gave a probability of 92% (AUROC = 0.92) with a statistical significance of <0,0001. Maximum Sensitivity was 80% and Specificity was 94%, with a cut-off of 24%.

Results obtained by the surrogate neutralizing assay were correlated with those of a pseudovirus neutralizing assay (PVNT) using a panel of 45 serum samples from adults vaccinated with the BNT162b2 vaccine at 5 timepoints - baseline, 1 week after first dose and 2, 8 and 20 weeks after the second dose ($r=0.83$; $p<0.0001$).

Supplementary figures

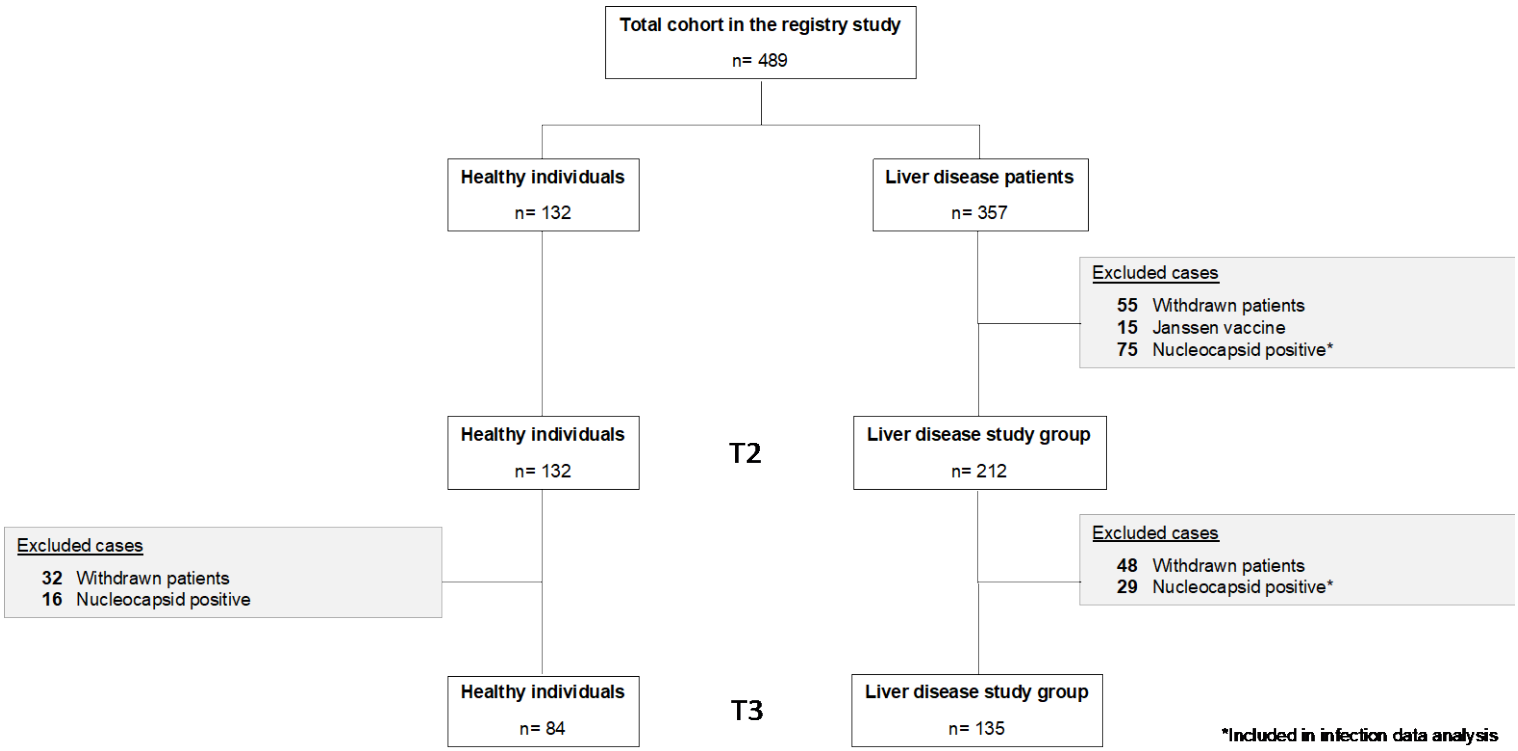

Fig. S1. CONSORT flow chart with the exclusion criteria and the study cohorts. T2 - two weeks after two dose-vaccination; T3 - six months after start of vaccination

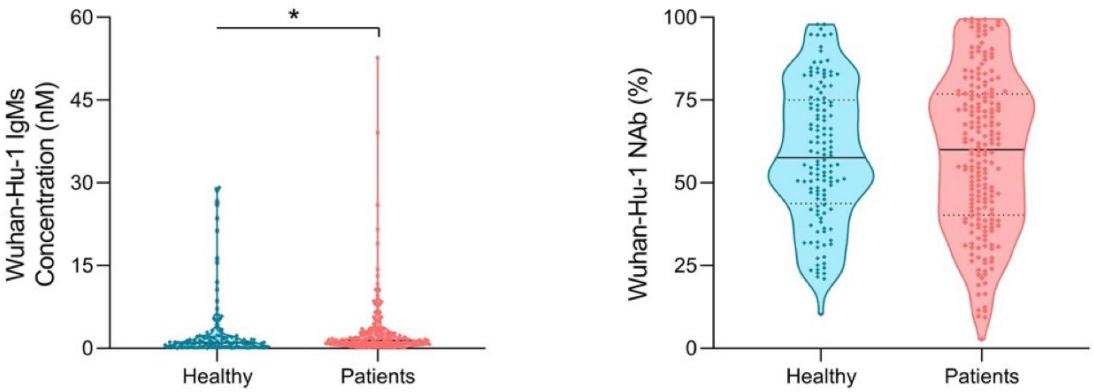

Fig. S2. IgM and NAb titers two-weeks after the second SARS-CoV-2 vaccine dose (T2) in healthy volunteers and patients with CLD. Violin plots representing IgG, IgM and NAb levels. Black horizontal lines indicate the median and grey dotted lines indicate the interquartile range. Parametric or non-parametric data was analyzed using the Student's t-test or the Mann Whitney test, respectively. *$p<0.05$

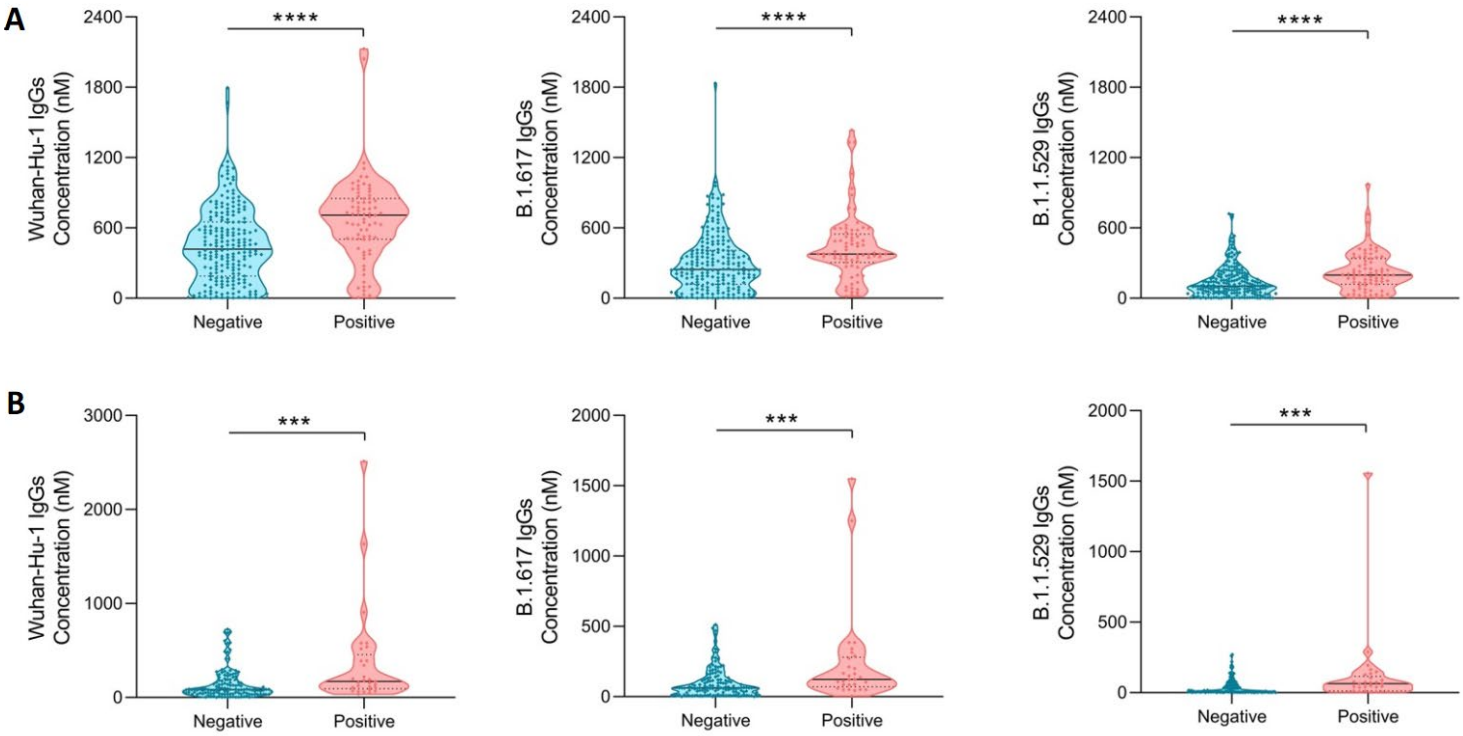

Fig. S3. Wuhan-Hu-1, B.1.617 (Delta) and B.1.1.529 (Omicron) IgG titers in COVID-19 non-infected versus priorly infected CLD patients. Violin plots representing IgG levels at (A) two-weeks after the second SARS-CoV-2 vaccine dose (T2) and (B) six months after the start of vaccination (T3). Black horizontal lines indicate the median and grey dotted lines indicate the interquartile range. Parametric or non-parametric data was analyzed using the Student's t-test or the Mann Whitney test, respectively. ***p<0.001; ****p<0.0001.

Supplementary tables

Table S1 Antibody response to COVID-19 vaccination in healthy volunteers and patients with CLD two weeks after the second SARS-CoV-2 vaccine dose (T2)

Healthy Volunteers	ChAdOx1 nCoV-19 (n = 12)	mRNA-1273 (n = 10)	BNT162b2 (n = 110)	Total (n = 132)	*p* value
Age	41.0 (33.0 – 55.3)	39.5 (27.8 – 59.5)	46.5 (38.0 – 58.0)	45.5 (37.0 – 57.3)	*ns*
Male	6 (50.0)	3 (30.0)	28 (25.5)	37 (28.0)	*ns*
Days after second dose vaccination	15.0 (15.0 – 15.0)	28.0 (16.8 – 28.0)	17.0 (15.0 – 21.0)	16.0 (15.0 – 21.0)	*ns*
Negative serologic response (IgG)	1 (8.3)	0 (0.0)	3 (2.7)	4 (3.0)	*ns*
Negative serologic response (NAb)	1 (8.3)	0 (0.0)	6 (5.5)	7 (5.3)	*ns*
Antibody titer (T2)					
IgG Wuhan-Hu-1 (nM)	276.7 (87.2 – 397.0)	768.4 (555.3 – 1,009.2)	408.9 (250.2 – 541.0)	409.6 (254.3 – 576.3)	*<0.0001*
IgM (nM)	0.4 (0.2 – 1.6)	12.0 (4.8 – 22.5)	1.0 (0.3 – 2.0)	1.0 (0.3 – 2.2)	*<0.0001*
% of neutralization	39.8 (33.0 – 49.8)	85.3 (76.3 – 94.7)	58.4 (46.1 – 74.1)	57.6 (43.8 – 75.1)	*<0.0001*
Patients with CLD	**ChAdOx1 nCoV-19 (n = 23)**	**mRNA-1273 (n = 40)**	**BNT162b2 (n = 149)**	**Total (n = 212)**	***p* value**
Antibody titer at baseline (T0)					
IgG Wuhan-Hu-1 (nM)	0.2 (0.0 – 0.6)	0.0 (0.0 – 0.6)	0.2 (0.0 – 1.1)	0.2 (0.0 – 0.9)	*ns*
IgM (nM)	0.2 (0.1 – 0.5)	0.2 (0.1 – 0.3)	0.3 (0.1 – 0.5)	0.3 (0.1 - 0.5)	*ns*
% of neutralization	17.8 (10.4 – 24.5)	10.5 (7.3 – 13.2)	17.1 (9.4 – 22.5)	14.8 (8.4 – 21.1)	*<0.01*
Days after second dose vaccination	19.0 (15.0 – 22.0)	17.0 (15.0 – 20.0)	15.5 (14.0 – 23.0)	17.0 (14.0 – 22.5)	*ns*
Negative serologic response (IgG)	0 (0.0)	1 (2.5)	7 (4.7)	8 (3.8)	*ns*
Negative serologic response (NAb)	3 (13.0)	5 (12.5)	8 (5.4)	16 (7.5)	*ns*
Antibody titer (T2)					
IgG Wuhan-Hu-1 (nM)	61.7 (40.7 – 206.1)	535.3 (310.8 – 856.1)	444.0 (242.7 – 649.8)	419.0 (188.1 – 650.0)	*<0.0001*
IgM (nM)	0.9 (0.6 – 1.7)	1.7 (0.9 – 5.1)	1.4 (0.8 – 2.8)	1.4 (0.8 – 2.8)	*<0.01*
% of neutralization	42.6 (36.2 – 53.6)	61.9 (35.1 – 85.9)	64.2 (44.2 – 77.0)	60.0 (40.3 – 76.9)	*<0.05*

Data are displayed as median (interquartile range) for continuous variables and number (%) for gender. Kruskal-Wallis and Pearson's Chi-square ($\chi 2$) test were used to compare quantitative and qualitative variables, respectively, between the three vaccine developers. $p < 0.05$ was considered statistically significant.

Table S2 Antibody response to COVID-19 vaccines in healthy volunteers and patients with CLD two weeks after two-dose vaccination (T2) and 6 months after start of vaccination (T3)

Antibody titer in healthy volunteers (T2)	**ChAdOx1 nCoV-19 (n = 12)**	**mRNA-1273 (n = 10)**	**BNT162b2 (n = 110)**	**Total (n = 132)**	***p* value**
IgG B.1.617 (nM)	174.2 (58.6 – 290.0)	454.8 (356.0 – 653.7)	275.5 (149.4 – 413.2)	275.5 (152.8 – 422.8)	*<0.01*
IgG B.1.1.529 (nM)	157.4 (32.3 – 214.2)	245.2 (113.9 – 409.1)	130.1 (86.4 – 220.8)	136.5 (89.3 – 224.6)	*<0.05*
Antibody titer in patients with CLD (T2)	**ChAdOx1 nCoV-19 (n = 23)**	**mRNA-1273 (n = 40)**	**BNT162b2 (n = 149)**	**Total (n = 212)**	***p* value**
IgG B.1.617 (nM)	47.1 (23.3 – 78.2)†	352.6 (214.1 – 571.6)	257.4 (143.9 – 410.0)	245.4 (119.9 – 408.2)	*<0.0001*
IgG B.1.1.529 (nM)	24.5 (4.7 – 78.5)‡	141.4 (79.7 – 295.8)	110.8 (56.4 – 188.8)	103.6 (47.4 – 192.6)†	*<0.0001*
Antibody titer in healthy volunteers (T3)	**ChAdOx1 nCoV-19 (n = 8)**	**mRNA-1273 (n = 2)**	**BNT162b2 (n = 74)**	**Total (n = 84)**	***p* value**
IgG Wuhan-Hu-1 (nM)	82.7 (50.0 – 127.2)	293.4 (238.8 - .)	80.8 (46.8 – 142.8)	85.0 (47.5 – 149.9)	*ns*
IgG B.1.617 (nM)	55.0 (43.0 – 90.2)	204.4 (156.2 - .)	48.9 (34.8 – 86.9)	50.9 (35.2 – 92.0)	*ns*
IgG B.1.1.529 (nM)	20.5 (13.8 – 37.5)	65.2 (62.0 - .)	17.6 (10.9 – 28.0)	17.8 (11.0 – 32.9)	*ns*
Days after start of vaccination	201 (198-201)	190 (190-192.5)	190 (170-195)	192 (173.8-196.3)	*ns*
Antibody titer in patients with CLD (T3)	**ChAdOx1 nCoV-19 (n = 18)**	**mRNA-1273 (n = 25)**	**BNT162b2 (n = 92)**	**Total (n = 135)**	***p* value**
IgG Wuhan-Hu-1 (nM)	32.8 (8.6 – 85.9)	176.1 (81.5 – 257.2)	82.0 (40.1 – 147.4)	83.1 (39.4 – 183.2)	*<0.0001*
IgG B.1.617 (nM)	14.6 (5.4 – 67.9)	98.8 (59.3 – 171.2)	61.4 (13.7- 113.0)	62.3 (14.7 – 120.8)	*<0.01*
IgG B.1.1.529 (nM)	4.0 (2.5 – 8.5)	16.2 (8.7 – 57.6)	8.6 (4.0 – 55.9)	9.4 (3.9 – 54.1)	*<0.05*
Days after start of vaccination	190 (182.5-206.5)	180 (179-182)	187 (181-209)	185 (180-208.5)	*ns*

Data are displayed as median (interquartile range). Kruskal-Wallis test was used to compare the variables between the three vaccine developers, and Mann-Whitney test to compare between healthy volunteers and CLD patients. †$p < 0.05$ and ‡$p < 0.01$ comparing with healthy volunteers from the same time-point.

Table S3 Demographic and clinical characteristics of CLD patients infected and non-infected with COVID-19 prior to two-dose vaccination (T2)

	Non-infected patients (n = 212)	SARS-CoV-2 infection (n = 75)	*p* value
General characteristics			
Age	57.0 (52.0 – 64.0)	57.0 (50.0 – 64.0)	*ns*
Male	120 (56.6)	44 (58.7)	*ns*
Ethnicity			
Caucasian	200 (94.3)	69 (92.0)	
Asian	4 (1.9)	2 (2.7)	
African	3 (1.4)	2 (2.7)	*ns*
Other	3 (1.4)	2 (2.7)	
Not reported	2 (0.9)	0 (0.0)	
Weight	75.0 (65.0 – 88.0)	78.0 (68.5 – 88.0)	*ns*
Height	170.0 (164.0 – 176.0)	171.0 (163.0 – 175.3)	*ns*
BMI (kg/m^2)	26.0 (23.1 – 30.3)	26.6 (23.7 – 30.4)	*ns*
Etiology of liver disease			
Viral Hepatitis	98 (45.3)	27 (36.0)	*ns*
Autoimmune and/or cholestatic liver disease	27 (12.7)	12 (16.0)	*ns*
Metabolic related fatty liver disease	97 (45.8)	33 (44.0)	*ns*
Hereditary liver disease	5 (2.4)	3 (4.0)	*ns*
Cryptogenic liver disease	1 (0.5)	1 (1.3)	*ns*
Pharmacotherapy			
Immunosuppressive treatment	18 (8.5)	6 (8.0)	
Antiviral therapy	25 (11.8)	7 (9.3)	
Metabolic therapy	70 (33.0)	26 (34.7)	
Stage of liver disease			
F3 – F4	141 (66.5)	50 (66.7)	*ns*
Liver cirrhosis	131 (61.8)	48 (64.0)	*ns*
Hepatocellular Carcinoma	15 (7.1)	1 (1.3)	*ns*
Comorbidities			
Type 2 diabetes mellitus	54 (25.5)	19 (25.3)	*ns*
Arterial Hypertension	66 (31.1)	29 (38.7)	*ns*
Obesity	42 (19.8)	16 (21.3)	*ns*
Hypertriglyceridemia	14 (6.6)	4 (5.3)	*ns*
Hypercholesterolemia	34 (16.0)	9 (12.0)	*ns*
Renal Insufficiency	8 (3.8)	4 (5.3)	*ns*
Asthma	9 (4.2)	2 (2.7)	*ns*
Heart/cardiovascular disease	26 (12.3)	10 (13.3)	*ns*
Smoking	26 (12.3)	12 (16.0)	*ns*
Other	51 (24.1)	12 (16.0)	*ns*
Vaccine type			
mRNA-1273	40 (18.9)	23 (30.7)	
ChAdOx1 nCoV-19	23 (10.8)	6 (8.0)	*ns*
BNT162b2	149 (70.3)	46 (61.3)	

Data are displayed as median (interquartile range) for continuous and number (%) for categorical variable. Mann-Whitney and Fisher's exact tests were used to compare between groups quantitative and qualitative variables, respectively. $p < 0.05$ was considered statistically significant.

Supplementary references

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